Expressed cDNAs from Embryonic and Larval Stages of the Horn Fly (Diptera: Muscidae)

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J. Med. Entomol. 45(4): 686–692 (2008)

ABSTRACT We used an expressed sequence tag approach to initiate a study of the genome of the horn fly, *Hematobia irritans* (L.) (Diptera: Muscidae). Two normalized cDNA libraries were synthesized from RNA isolated from embryos and first instars from a field population of horn flies. Approximately 10,000 clones were sequenced from both the 5′ and 3′ directions. Sequence data from each library was assembled into a database of tentative consensus sequences (TCs) and singletons and used to search public protein databases and annotate the sequences. Additionally, the sequences from both the egg and larval libraries were combined into a single database consisting of 16,702 expressed sequence tags (ESTs) assembling into 2886 TCs and 1,522 singleton entries. Several sequences were identified that may have roles in the horn fly's resistance to insecticides. The availability of this database will facilitate the design of microarray and other experiments to study horn fly gene expression on a larger scale than previously possible. This would include studies designed to investigate metabolic-based insecticide resistance, identify novel antigens for vaccine-based control approaches, and discover new proteins to serve as targets for new pesticide development.

KEY WORDS *Hematobia irritans*, expressed sequence tags, insecticide resistance, cytochrome P450, esterase

The horn fly, *Hematobia irritans* (L.) (Diptera: Muscidae), is an obligate blood-feeding parasite of cattle, and control of these flies is a major concern to cattle producers in North and South America. The horn fly affects the productivity of cattle by interfering with normal feeding activity, causing loss of blood, reduced weight gains, and decreased milk production in infested mother cows. Economic losses to producers in the United States and Brazil have been estimated at US$876 and US$150 million annually, respectively (Kunz et al. 1991, Grisi et al. 2002). The primary means of controlling the horn fly is through the use of insecticides, primarily pyrethroids and organophosphates. However, resistance to insecticides of these two classes of compounds is becoming common through many parts of the range of the horn fly (Kunz and Schmidt 1985, Kunz et al. 1995, Guerrero and Barros 2006). A major effort in horn fly research relates to maximizing the effectiveness of insecticide treatment protocols and extending the usefulness of current classes of insecticides (Barros et al. 1999, Byford et al. 1999, Oremus et al. 2006). The development of novel horn fly control methodologies would be an important contribution for the cattle industry.

Genome sequencing projects have revolutionized the study of biological organisms, and they can provide new insight and opportunities to solve problems relating to human health and agriculture. We expect the development of novel horn fly control methodologies to be advanced by knowledge of the expressed gene coding regions of the fly. This knowledge can guide searches for new chemotherapeutics for pesticide development or antigenic molecules that can provide a vaccine-based fly control strategy. Little has been published about the genomics or molecular biology of the horn fly, although the karyotype of a laboratory-reared population was determined to consist of five homomorphic chromosome pairs (Avancini and Weinzierl 1994). The genome size was reported as 2.2×10^9 bp through a personal communication noted by Robertson and Lampe (1995); these researchers also reported the horn fly genome contains an extremely high number of *mariner* elements, up to 1% of the genome or ≈17,000 copies. The relatively large size estimate for the horn fly genome diminishes the likelihood of obtaining whole genome sequence in the

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near future, thus expressed sequence tag (EST)-based projects will likely be more feasible approaches to study the genome and molecular biology of this fly. Currently, there are very few GenBank entries for the horn fly. Only 86 horn fly records were found in the nr database and half of these were mitochondrial (15) or transposase-related (28) entries. There are 221 EST entries, with 214 coming from the submissions of Guerrero et al. (2004) from their various studies of horn fly genes involved in diapause induction in early larval stages or insecticide resistance in adult stages. As a component of a project directed toward transformation of the horn fly with embryonic-expressed gene products, we have developed an EST database of genes expressed in the embryo and first instar larvae of the horn fly. Because of the need to control these pests in situations where insecticide resistance exists, we have examined the EST database for sequences with similarity to insecticide resistance-associated genes from other insect species. We hope to deepen our understanding of pyrethroid and diazinon resistance mechanisms, particularly metabolism-based resistance. Metabolic resistance has been well documented in the horn fly (Sheppard 1995, Guerrero and Barros 2006); however, the molecular basis has not been established, and there are very few sequences from candidate pesticide-metabolizing enzymes from the horn fly (e.g., esterases or cytochromes P450) in GenBank. Augmentation of the list of candidate metabolic resistance genes from the horn fly is an additional major aim of this EST database project.

Materials and Methods

Fly Rearing. Adult flies were collected from pastured cattle at the Louisiana State University Agricultural Center St. Gabriel Research Station (St. Gabriel, LA) by aerial nets and held in Erlenmeyer flasks at 30°C and total darkness for 1.5 h to facilitate egg collection (Lysyk 1991). The collected eggs were divided into two samples, and the adult flies were frozen at −80°C. One egg sample was frozen at −80°C, and the second egg sample was transferred to moist filter paper in petri plates and maintained at room temperature. The following day, the wandering first instars were collected and frozen at −80°C.

Nucleic Acid Manipulations. Total RNA was isolated from 0.17 and 0.09 g of egg and first instar material, respectively, by using two consecutive 40-s runs in the FastPrep-24 Tissue and Cell Homogenizer (Qbiogene, Irvine, CA). A setting of 6.0 with a 3-min ice incubation between runs was used, followed by RNA isolation with the Fast RNA Pro Green kit with Lysing Matrix D (Qbiogene) and subsequent lithium chloride precipitation using the Totally RNA kit reagents (Ambion, Austin, TX). RNA integrity was verified by formaldehyde gel electrophoresis and staining in GelStar Nucleic Acid Gel Stain (Lonza Rockland, Inc., Rockland, ME). Total RNA was used to construct a standard normalized cDNA library under contract by Express Genomics, Inc. (Frederick, MD), by using proprietary protocols. Briefly, cDNA synthesis was primed using an oligo-dTVN primer and double-stranded cDNA directionally cloned into the EcoRV-NotI site of pExpress1 after size selection on an agarose gel. cDNA normalization was carried out using biotinylated RNA driver transcribed from the cloned cDNA and hybridization with single-stranded cDNA circles generated by phagemid production. After hybrid removal using streptavidin, single-stranded circles were converted into double stranded DNA and then electroporated into T1 phage resistant DH10b *Escherichia coli* cells.

EST sequencing was performed at the J. Craig Venter Institute (Rockville, MD). Bacterial colonies were picked for template preparation using colony-picking robots (Genetix, Boston, MA), inoculated into 384-well plates containing liquid medium and grown overnight. A robotic workstation was used to prepare sequencing grade plasmid DNA by using an alkaline lysis method (Sambrook et al. 1989) modified for high-throughput processing. Beckman Multimek 96 or Biomek FX automated pipetting robot workstations (Beckman Coulter, Fullerton, CA) were used to combine prealiquoted templates and reaction mixes consisting of deoxy- and fluorescently labeled dideoxynucleotides, *Taq*DNA polymerase, sequencing primers, and sequencing reaction buffer. Linear amplification steps were performed on MJ Research Tetrads PTC-225 (MJ Research, Inc., Watertown, MA) and sequencing reaction products purified by ethanol precipitation and resolved on ABI 3730xl sequencing machines (Applied Biosystems, Foster City, CA). Each clone was assigned a seven-digit label with a unique library identifier of "EGG" or "LAR," which varied from EGGA001TF to EGGBG95TR and LARV002TF to LARWG96TR. The unassembled EST sequences were submitted to GenBank dbEST with the accession numbers of FD449556–FD457982 for the egg-derived ESTs and FD457983–FD466257 for the larval-derived ESTs.

Bioinformatic Analysis of ESTs. The EST sequences were mapped to functional classifications schemes such as Gene Ontology (GO) terms (Ashburner et al. 2000), KEGG pathways (Ogata et al. 1999), and Swiss Prot Protein Keywords (Bairoch et al. 2005), through the use of High Throughput Gene Ontology and Functional Annotation Toolkit (HT-GOFAT; http://liru.ars.usda.gov), which is a functional annotation engine based upon WND-BLAST (Dowd et al. 2005) and the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Dennis Jr. et al. 2003).

Results

To facilitate gene discovery and gene expression studies in embryonic and early larval stages of the horn fly, we synthesized two normalized cDNA plasmid libraries from total RNA isolated from horn fly eggs and first instars. From 0.17 and 0.09 g of eggs and first instars, we obtained 420 and 150 μg of total RNA, respectively. The initial non-normalized egg cDNA

Table 1. Ten most abundant assembled sequences from EGG Library and LARVAL Library, and combined assembled data set

ID	ESTs no.	bp	Top BlastX Hit		
			ID	Accession no.	e value
EGG library data set					
Contig 82[a]	74	1,303	Horn fly mitochondrial sequence	DQ029097	0
Contig 1061	29	789	Small RNP Particle Protein B	Q05856	1.7e-63
Contig 265	27	877	NADH Dehydrogenase (Ubiquinone) Fe-S Protein 8	P42028	9.0e-88
Contig 91	21	1,192	H3 Histone	Q6P823	3.4e-70
Contig 315	21	819	Big Brother	Q24040	8.6e-98
Contig 1531	21	879	Oligomycin Sensitivity-Conferring Protein	Q24439	3.3e-82
Contig 14	20	756	Probable Fatty Acid-Binding Protein	Q17017	2.6e-29
Contig 184	20	1,020	Histone H2A Variant	P08985	3.3e-55
Contig 418	20	540	40S Ribosomal Protein S30	P62864	1.8e-13
Contig 22	19	941	Signal Sequence Receptor Subunit γ	Q9UNL2	1.2e-61
Larval library data set					
Contig 166[a]	155	1,303	Horn fly mitochondrial sequence	DQ029097	0
Contig 22	25	680	Probable Fatty Acid-Binding Protein	Q17017	2.1e-29
Contig 780	25	928	Vacuolar H^+ ATPase G-Subunit	Q9XZH6	4.1e-25
Contig 85	24	1,356	NADH:Ubiquinone Reductase 42-kDa subunit Precursor	P91929	1.6e-170
Contig 37[a]	23	1,680	*Drosophila melanogaster* CG9894-PA	NM_164498.1	9e-22
Contig 217	23	1,455	Ferritin Light Chain	P19976	7.7e-14
Contig 253	22	931	Amyloid β (A4) Precursor-like Protein 2	Q06481	4.0e-12
Contig 58[a]	21	2,260	18S Ribosomal RNA gene	EU179518.1	0
Contig 74	19	1,155	Adenylate Kinase 2	Q9WTP6	1.1e-86
Contig 238	19	946	Muscle LIM protein	P53777	2.7e-40
Combined assembled data set					
Contig 165[a]	229	1,303	Horn fly mitochondrial sequence	DQ029097	0
Contig 36	45	766	Probable Fatty Acid-Binding Protein	Q17017	2.6e-29
Contig 1295	39	990	Vacuolar H^+ ATPase G-Subunit	Q9XZH6	4.4e-25
Contig 495	39	878	NADH Dehydrogenase (Ubiquinone) Fe-S Protein 8	P42028	9.0e-88
Contig 70	38	1,200	H3 Histone, family 3B	Q6P823	3.5e-70
Contig 418	35	1,097	Peptidyl-Prolyl Cis-Trans Isomerase 5 Precursor	P52013	3.4e-69
Contig 64[a]	33	1,679	*Drosophila melanogaster* CG9894-PA	NM_164498.1	9e-22
Contig 1039	32	789	Small RNP Particle Protein B	Q05856	1.7e-63
Contig 862	31	879	ATP Synthase O Subunit Mitochondrial Precursor	Q24439	1.3e-81
Contig 95[a]	31	2,283	18S Ribosomal RNA gene	EU179518.1	0

[a] Data obtained from BlastN search of GenBank nr database.

library consisted of a total of 7.2×10^7 colony-forming units (cfu), and after normalization, 6.6×10^7 cfu, 87% recombinants were recovered. The average insert size was 1.1 kb, and there was a 120-fold reduction in clones that hybridized to an actin probe compared with the primary library. The initial non-normalized first instar cDNA library consisted of a total of 1.5×10^7 cfu and after normalization, 1.8×10^8 cfu, 96% recombinants, were recovered. The average insert size was 1.05 kb, and there was a 150-fold reduction in clones that hybridized to an actin probe compared with the primary library.

Sequencing templates were prepared from 10,224 clones, and each clone was sequenced with both the pUC/M13Reverse and pUC/M13Forward primers. The average length of the egg and larval ESTs was 586 and 612, respectively. There were 8,427 egg-derived ESTs that assembled into 2,668 sequences, with an average length of 814 bases, whereas the 8,275 larval-derived ESTs assembled into 2,689 sequences, with an average length of 855 bases. Combining data from both libraries and assembling into one database, a total of 9.9 Mb of sequence data were accumulated from 16,702 egg and larval ESTs (3,746 reactions did not yield usable sequence data). These ESTs assembled into a dataset consisting of 2,886 tentative consensus sequences (TCs) and 1,522 singletons, with an average read length of 864 bases. Individual members of the EST sequence database were used to search public protein databases using BLASTX routines and assigned putative function. These searches were able to assign significant similarities (e value <0.001) to 1,948 (73%) and 1,788 (67%) of the 2,667 egg and 2,688 larval sequences, respectively. See Supplementary Material for assembled egg and larval and combined databases and annotations. Before our study, 307 horn fly sequences were present in GenBank. Bioinformatic analysis using BlastX found that 90 of the 307 preexisting sequences have significant matches to members of our horn fly database (e value <1 e-50). Table 1 lists the characteristics of the top 10 most abundant ESTs from each library, with the caveat that these libraries were normalized, reducing the utility of the abundance data for predicting quantitative gene expression. The most abundant contig in each of the three assembled data sets was the same sequence, although a BlastX hit was not found during the search of the protein databases. Thus, the GenBank nr database was searched with the BlastN algorithm that found an identity with a 1,300-bp sequence from the horn fly mitochondria (accession no. DQ029097). Two other entries that both occurred in the larval and combined data set necessitated similar analyses. The BlastN analyses for these two entries found sequence similarity to *Drosophila melanogaster* (Meigen) CG9894-PA and 18S Ribosomal RNA gene (Ta-

Table 2. Occurrence of GO terms (biological process) related to embryonic and larval development and functions

GO term	Egg library count	Larval library count
Axis specification	15	15
Cell fate commitment	24	0
Cell fate determination	13	0
Central nervous system development	14	0
Cystoblast division	4	0
Determination of adult life span	8	0
Development	139	141
Embryonic development	36	32
Embryonic development sensu Insecta	19	17
Embryonic development sensu Metazoa	22	19
Embryonic morphogenesis	12	0
Gametogenesis	59	53
Gland development	15	0
Larval or pupal development sensu Insecta	0	37
Larval development sensu Insecta	0	6
Larval cuticle biosynthesis sensu Insecta	0	3
Larval development	0	7
Morphogenesis of an epithelium	14	14
Morphogenesis of an embryonic epithelium	11	0
Nervous system development	51	41
Neurogenesis	19	18
Stem cell division	7	0

ble 1). To confirm that our libraries contained stage-specific expressed sequences, we enumerated the ESTs from each library that contained Biological Process GO terms related to several embryonic and larval development and functions (Table 2). The tabulated GO term frequencies are consistent with library source. For example, the GO Terms Cell fate commitment, Cell fate determination, Central nervous system development, Cystoblast division, Determination of adult life span, Embryonic morphogenesis, Morphogenesis of an embryonic epithelium, and Stem cell division were found exclusively in the egg library database. Conversely, the GO Terms Larval or pupal development sensu Insecta, Larval development sensu Insecta, Larval cuticle biosynthesis sensu Insecta, and Larval development were strictly found in the larval library database entries. GO Terms that were expected to be involved in both larval and embryonic stages, such as Morphogenesis of an epithelium, Nervous system development, and Neurogenesis, were found equally distributed in both library data sets.

Focusing on the problems in controlling this cattle pest, we examined the combined EST database for expressed genes that might play roles in this fly's resistance to pesticides. Esterases, cytochromes P450, and glutathione transferases are enzyme families with diverse metabolic functions, and some family members have roles in the metabolism or sequestration of pesticides. Table 3 lists sequences from the combined assembled EST database whose putative identity from BlastX analysis (e value <0.001) was a member of one of these gene families. The egg library contained one esterase, one cytochrome P450, and three glutathione transferase sequences. The larval library contained three esterase, nine cytochromes P450, and seven glutathione transferase sequences. Furthermore, because of the appeal of G protein-coupled receptors as potential drug targets for development by the pharmaceutical industry, we examined the annotation of ESTs

Table 3. Possible insecticide resistance-associated sequences identified in combined assembled EST database

ID	bp	Top BlastX Hit			Library
		ID	Accession no.	e value	
Esterases					
Contig 2728	1,942	Esterase D/formylglutathione hydrolase	Q9R0P3	7.4e-99	E + L
Contig 3187	829	Esterase B1 precursor	P16854	8.6e-45	L
Contig 3520	460	Esterase B1 precursor	P16854	1.9e-3	L
Cytochrome P450s					
Contig 3157	710	P450 family 6A9	Q27594	5.2e-66	L
Contig 3515	616	P450 family 6A13	Q9V4U9	8.2e-43	L
Contig 364	951	P450–4D1	P33269	6.4e-82	L
Contig 772	576	P450 4D10	O18596	6.4e-84	L
Contig 78	804	P450 6A9	Q27594	1.6e-72	L
Contig 850	1,320	P450 4C3	Q9VA27	1.6e-116	L
Contig 1670	1,183	P450 4G1	Q9V3S0	3.2e-108	L
Contig 2077	1,320	P450 71D7	P93531	2.0e-77	E
Contig 233	1,233	P450 6A9	Q27594	1.0e-133	L
Contig 270	1,670	P450 6A9	Q27594	0	L
Glutathione transferases					
Contig 1587	703	Microsomal GST 1	P10620	4.1e-23	E + L
Contig 2016	673	GST D1	P20432	1.5e-75	L
Contig 2286	972	GST θ 1	Q64471	1.0e-34	L
Contig 2775	718	GST 1	P28338	1.8e-90	E + L
Contig 287	806	GST 1	P28338	5.3e-100	E + L
Contig 732	642	GST1–1	P42860	1.3e-73	L
Contig 757	1,054	GST	P46437	2.8e-113	L
Miscellaneous					
Contig 2021	349	GABA receptor modulator	P07108	2.8e-6	L
Contig 99	1,034	GABA receptor-associated protein	P60517	4.7e-57	E + L

from the egg and larval databases whose molecular function GO terms included G protein-coupled receptor. One promising candidate G protein-coupled receptor was found in the larval library, sequence 772, which is 972 bp and has significant sequence similarity to Angiotensin II Receptor-Associated Protein (BlastX e-value 8.8 e-10) from *Rattus norvegicus*, accession no. Q642A2.

Discussion

These databases were established to serve as a foundation for a project looking at embryonic and early larval gene expression. As an indication of the stage specificity of ESTs from each library, we examined Biological Process GO term annotation of ESTs from each library, and we found the occurrence of embryonic and larval development- or function-related GO terms was consistent with the fly stage RNA source used to synthesize the library. These GO Term distribution results were reassuring that these cDNA samples, and the resulting embryonic and larval sequence data sets can form the basis of microarrays designed for future projects to quantitate and verify overall and specific gene expression in embryos and early stage larvae of the horn fly. We are exploring the feasibility of using these cDNAs with pyrosequencing technologies to obtain a deeper sampling of embryonic and larval expressed genes and expanding the databases reported in this study.

We also wanted to use the data to contribute to a better understanding of insecticide resistance mechanisms in this common livestock pest, because this is another research interest in our group. The primary insecticides used to control this fly are pyrethroids and organophosphates, which target the sodium channel (Catterall 1995) and acetylcholinesterase (Aldridge 1950), respectively. Specific sodium channel gene mutations that lead to pyrethroid resistance in the horn fly have been isolated and characterized in field populations from the United States (Guerrero et al. 1997, 2002). Recently, a gene sequence for horn fly acetylcholinesterase was submitted to GenBank (Temeyer and Chen 2007), although organophosphate resistance-associated mutations in this gene have not been reported despite the occurrence of organophosphate resistance in field populations. This might be due to the fly developing other mechanisms of OP resistance besides insensitive acetylcholinesterase. Besides target site insensitivity, other well-established mechanisms of insecticide resistance that have been documented in the horn fly involve detoxification or sequestration by metabolic esterases (Guerrero et al. 1999, Guerrero 2000, Li et al. 2007) or mixed function oxidases (Sheppard 1995, Guerrero et al. 1997) such as cytochromes P450. A third family of enzymes that can be involved in pesticide resistance are the glutathione transferases, although their involvement in the resistance of the horn fly to pesticides has not been reported. Several of the orthologs of the possible metabolic resistance-associated gene sequences of Table 3 have been implicated in resistance mechanisms of various dipterans. For example, the top BlastX hit for sequences 3187 and 3520 is Esterase B1, an esterase that has been associated with organophosphate resistance in several *Culex* species (Mouches et al. 1990). Sequences 3187 and 3520 were found only in the larval library and the expression of the corresponding Esterase B1 transcript might play a role in xenobiotic survival of larvae in the manure pat where larval feeding and maturation and pupal development takes place. The top BlastX hit for sequences 3157, 3515, 78, 233, and 270 are members of the cytochrome P450 family 6, members of which have been associated with insecticide resistance in *Musca domestica* (L.) (Tomita and Scott 1995), *Helicoverpa armigera* (Hübner) (Ranasinghe and Hobbs 1998), and *Anopheles gambiae* Giles (Nikou et al. 2003). The cytochrome P450 family is well known as an enzyme family capable of detoxification of a wide range of xenobiotics, often with very specialized family members. Like the Esterase D noted above, these P450 family 6-like sequences also were isolated from the larval cDNA library and not observed in the data from the embryonic library. It should be noted that most, if not all, horn fly insecticide resistance bioassays are performed on the adult life stage, and the RNA used to synthesize our libraries did not originate from the adult stage. However, it is possible that a number of the genes noted in Table 3 are expressed across several life stages of the fly. For example, the top BlastX hit for sequence 2728 is Esterase D, an enzyme that is expressed in many human tissues and cell types (Hopkinson et al. 1973). Also, sequence 364 shows significant similarity to P450-4D1, which has been studied in *D. melanogaster* and found to be expressed through all stages of development in that species, with a peak in the late larval stages (Gandhi et al. 1992). Some of the glutathione transferases identified in the horn fly databases may play a role in insecticide resistance. Sequences 2016 and 732 are very similar to a glutathione transferase D1 from *D. melanogaster*, which has a role in DDT metabolism (Tang and Tu 1994). It is hoped that identification of these putative metabolic resistance-associated genes will assist our understanding of metabolic resistance mechanisms, which should help in the design of better control methodologies and programs against this economically significant pest of cattle. The acquisition of these expressed sequences from enzyme families associated with resistance in other dipterans will facilitate the design of microarrays and experiments to investigate metabolic resistance mechanisms in the horn fly. This horn fly database also should be valuable as a source of gene coding region sequences, which might prove amenable as antigens for a vaccine-based control approach. Cupp et al. (2004) have evaluated thrombostasin, an anti-thrombin peptide found in horn fly saliva, as a vaccine candidate to disrupt horn fly blood feeding. Bioinformatic analysis of this horn fly EST database could identify other candidates that could eventually lead to novel control methods against this pest.

Acknowledgments

We thank Cole Younger for collection of horn flies and the sequencing core personnel at the JCVI Joint Technology Center.

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Received 4 February 2008; accepted 7 April 2008.